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Suppressive effect of rebamipide, an antiulcer agent, against activation of human neutrophils exposed to formyl-methionyl-leucyl-phenylalanine

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Summary. Rebamipide, an antiulcer agent, has been shown to be able to prevent gastric mucosal injury resulting in part from activation of neutrophils. The mechanism of its suppressive action, however, remains to be established. The present study aimed to determine the effect of rebamipide on activation of isolated human neutrophils and to identify the signal transduction pathway involved in its regulation. In unstimulated cells, alkaline phosphatase activity was found residing in short rod-shaped intracellular granules. Upon stimulation with a chemotactic peptide formyl-methionyl-leucyl-phenylalanine, the granules fused to form elongated tubular structures and spherical vacuoles. Rebamipide inhibited reorganization of alkaline phosphatase-containing granules along with upregulation of alkaline phosphatase activity and CD16, a marker of the granules. It also suppressed chemotaxis, an increase in intracellular calcium ion concentration, and NADPH oxidase activation in cells stimulated with formyl-methionyl-leucyl-phenylalanine. In contrast, the drug showed no inhibitory action toward upregulation of alkaline phosphatase activity and CD16, and activation of NADPH oxidase in cells stimulated with phorbol myristate acetate, an activator of protein kinase C. These findings demonstrate that rebamipide exerts a broad spectrum of suppressive actions toward biological functions of human neutrophils stimulated with formyl-methionyl-leucyl-phenylalanine, but not with phorbol myristate acetate, and suggest that the upstream point of protein kinase C is the signal transduction pathway involved in its regulation.

Key words: Alkaline phosphatase, CD16, Chemotaxis, NADPH oxidase, Rebamipide

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Introduction

Rebamipide (2-(4-chlorobenzoylamino)-3-[2-(1H)-quinolinon-4-yl]propionic acid) is known to affect various biological functions and was shown as a preventer of the damage of gastric mucosa. The chemical inhibits nitric oxide production in murine macrophages (Nagano et al., 1998) and interleukin-8 release in gastric cancer cell lines (Aihara et al., 1998). It also induces production of substances essential for reparation of the gastric mucosa (for review see Majumdar et al., 1997), such as epidermal growth factor, glycosaminoglycan, and prostaglandin E_2 (Song et al., 1998; Takaishi et al., 1998; Tarnawski et al., 1998). In neutrophils, rebamipide inhibits production of reactive oxygen species and binding of formyl-methionyl-leucyl-phenylalanine (FMLP) to cell surface (Ogino et al., 1992; Yoshikawa et al., 1993; Naito et al., 1994, 1995; Yoshida et al., 1996; Kim and Hong, 1997; Danielsson and Jurstrand, 1998; Sakurai et al., 1998; Yamasaki et al., 1999).

Formation of gastric ulcer is associated with activation of neutrophils (Smith et al., 1987; Ogino et al., 1992), which tend to accumulate in particular areas of the gastric mucosa (Steer, 1985; Watanabe et al., 1997). Activation of neutrophils is accompanied by the release of superoxide. It is produced by the function of NADPH oxidase and then subsequently converted to reactive oxygen species, including hydrogen peroxide, singlet oxygen and hydroxyl radicals which participate in microbial killing and cause injury of surrounding tissue (for reviews see Robinson and Badway, 1995; Babior, 1999; Kobayashi and Seguchi, 1999).

In human neutrophils, superoxide is produced in a special type of intracellular compartments, alkaline phosphatase (ALPase)-positive secretory granules (Kobayashi et al., 1998). Upon cell stimulation, secretory granules fuse to form elongated tubular structures and vacuoles which eventually associate with the plasma membrane causing release of superoxide outside the cell (Kobayashi et al., 1998, 1999;

Kobayashi and Seguchi, 1999). These compartments contain membrane proteins involved in inflammatory reaction (Borregaard et al., 1993; Borregaard and Cowland, 1997).

In the present study, we evaluated the suppressive effect of rebamipide on reorganization of secretory granules, upregulation of distinct markers of these granules, ALPase activity and CD16, chemoattractant-directed migration, an increase in intracellular calcium ion concentration, and NADPH oxidase activation in isolated human neutrophils. A signal transduction pathway involved in regulation of the action of the drug is suggested.

Materials and methods

Reagents

Cytochrome c, dextran (av. MW = 520,000), Histopaque 1083, N-formyl-methionyl-leucyl-phenyl-alanine (FMLP), p-nitrophenylphosphate, phorbol 12-myristate 13-acetate (PMA), poly-l-lysine, superoxide dismutase, N-tris[hydroxymethyl]glycine (Tricine) and N-tris[hydroxymethyl]methyl-3-aminopropane-sulfonic acid (TAPS) were purchased from the Sigma Chemical Co. (St Louis, MO, USA). Rebamipide was a kind gift from Otsuka Pharmaceuticals, Co. Ltd. (Tokushima, Japan). Anti-CD16 was obtained from the Novocastra Lab. Ltd. (Newcastle, UK). Fluorescein-conjugated goat-IgG to mouse IgG was obtained from ICN Pharmaceuticals, Inc. (Aurora, OH, USA). Fura 2-AM was purchased from Molecular Probes, Inc. (Eugene, OR, USA). All other reagents were of the highest grade of purity available. Stock solutions of FMLP, PMA and Fura 2-AM were prepared in dimethyl sulfoxide (DMSO) and stored at -20 ºC.

Isolation of neutrophils

After obtaining an informed consent, human neutrophils were isolated from peripheral blood of healthy volunteers as previously described (Kobayashi and Robinson, 1991). Briefly, leukocytes were separated from erythrocytes by sedimentation in 6% dextran with acid citrate followed by enrichment centrifugation through Histopaque. Residual erythrocytes were removed by hypotonic lysis with distilled water. Cell viability was at least 97% as determined by trypan blue exclusion and purity of neutrophils was 95% by differential counting. Neutrophils were maintained in PBS until use.

Loading with rebamipide and cell stimulation

Cells ($1x10^6$ cells/ml) were exposed to 2 mM rebamipide in PBS containing 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 mM glucose for 10 min at 37 ºC followed by stimulation with either 10^{-7} M FMLP or 50 ng/ml PMA for 5 min at 37 ºC. In some experiments, cells were treated with various concentrations of rebamipide to examine their dose-dependency. Stock solutions of FMLP and PMA were diluted with DMSO so that the final concentration of the solvent in cell suspension was 0.25% (v/v). Unstimulated cells, serving as control, were incubated in a similar manner, but without addition of either FMLP or PMA.

Enzyme cytochemistry for ALPase detection

After loading with rebamipide followed by stimulation, cell suspensions were immediately mixed with an equal volume of PBS, containing 4% glutaraldehyde, and fixed for 5 min on ice followed by centrifugation. The resulting pellet was resuspended in PBS, cells were attached onto glass cover slips coated with 0.2% poly-l-lysine (Robinson, 1991) and placed in cell culture multiwell plates. Fixed cells were rinsed with 100 mM TAPS buffer (pH 9.4) and then subjected to cytochemical detection of the sites of ALPase activity (Kobayashi and Robinson, 1991). Briefly, cells were incubated in a reaction medium (pH 9.4) containing 100 mM TAPS, 50 mM Tricine, 2 mM p-nitrophenyl-phosphate, 2 mM $CeCl_3$, 2 mM $MgSO_4$, 0.006% Triton X-100, 0.004% saponin and 5% sucrose, for 1 hr at 37 °C, followed by washing with TAPS buffer.

Microscopy

After cytochemical incubation, cells were washed with 0.1M cacodylate buffer (pH 7.4). Postfixation was performed with 1% osmium tetroxide in cacodylate buffer for 30 min on ice followed by a rinse with cacodylate buffer. Cells were then dehydrated through a graded series of ethanols and embedded in Spurr's epoxy resin (Spurr, 1969). Blocks were cut with a Reichert OmU4 (Reichert-Jung; Vienna, Austria). Semi-thin (0.5 μm in thickness) sections were examined under a transmission electron microscope (JEM-100S; JEOL, Tokyo, Japan) operated at an accelerating voltage of 80 kV. For scanning electron microscopy, osmium tetroxide-fixed cells were treated with 1% tannic acid (Yamasaki et al., 1997), dehydrated in a graded series of ethanols, dried in a critical point dryer (Hitachi HCP-2; Hitachi, Tokyo, Japan) with liquid CO_2 and then coated with Pt (5 nm in thickness) in an ion-coater (Eiko IB-5; Eiko, Tokyo, Japan). The observation was done with a field emission scanning electron microscope (Hitachi S-700; Hitachi, Tokyo, Japan) operated at an accelerating voltage of 15 kV.

Measurement of ALPase activity

The amount of ALPase activity upregulated to the cell surface was determined spectrophotometrically by measuring the conversion of p-nitrophenylphosphate to p-nitrophenol (Kobayashi and Robinson, 1991). Fixed cells ($1x10^7$ cells/ml) were incubated for 15 min at 37 ºC

in a reaction medium as described above with the exception that cerium ions and detergents were not used. Reaction was stopped by transferring tubes containing cell suspension into an ice-cold water bath. Cellular materials were removed by rapid centrifugation. Resulting supernatant was read at 410 nm.

Flow cytometry of CD16

Cells were fixed with PBS containing 2% paraformaldehyde for 30 min on ice followed by washing in PBS. Fixed cells were incubated in PBS containing 5% normal goat serum and then exposed to mouse anti-CD16 IgG for 30 min at 37 °C followed by washing in PBS. The cells were further incubated in PBS containing fluorescein-conjugated goat IgG to mouse IgG for 1 hr at room temperature. Fluorescence intensity was analysed in a flow cytometer (FACScan; Becton Dickinson, San Jose, CA, USA).

Chemotaxis assay

Chemotaxis was assayed using a chemotaxis chamber (Chemotaxicell; Kurabo, Osaka, Japan) partitioned by a micropored polycarbonate membrane (diameter of pore 5 μm). Upper and lower chambers were filled with PBS containing Mg^{2+}, Ca^{2+}, glucose and rebamipide. Neutrophils ($1x10^5$ cells/ml), exposed to rebamipide for 10 min, were added to the upper chamber. Then, 10^{-7} M FMLP was added to the lower chamber followed by incubation for 10 min at 37 °C. After incubation, cells were immediately fixed with 2% paraformaldehyde in PBS and stained with toluidine blue. Micrographs of neutrophils were taken (forty micrographs from four separate experiments) and then migrating cells were counted in a random-sampling manner to determine their number.

Measurement of intracellular calcium ion concentration ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was assayed according to Whyte et al. (1993). Briefly, cells were incubated for 15 min at 37 °C in PBS containing Mg^{2+}, Ca^{2+}, glucose and 5 μM Fura 2-AM. Fluorescence was determined with a spectrofluorophotometer (Shimadzu RF-5300PC; Shimadzu, Kyoto, Japan) employing a dual wavelength excitation (340 and 380 nm) and emission at 510 nm. $[Ca^{2+}]_i$ was calculated on the basis of R_{max} (obtained by lysing cells with 1% Triton X-100) and R_{min} (obtained by adding 40 mM EGTA to lysed cells).

Measurement of NADPH oxidase activity

Superoxide release was measured spectrophotometrically employing a superoxide dismutase-inhibitable reduction of ferricytochrome c (McCord and Fridovich, 1969). The extent of cytochrome c reduction was determined by the change in absorbance at 550 nm with a spectrophotometer (Hitachi 220A; Hitachi, Tokyo, Japan). The rate of increase in A550 was used to calculate the cytochrome c reduction based on a specific absorbance of 21.1/mM/cm (Massey, 1959).

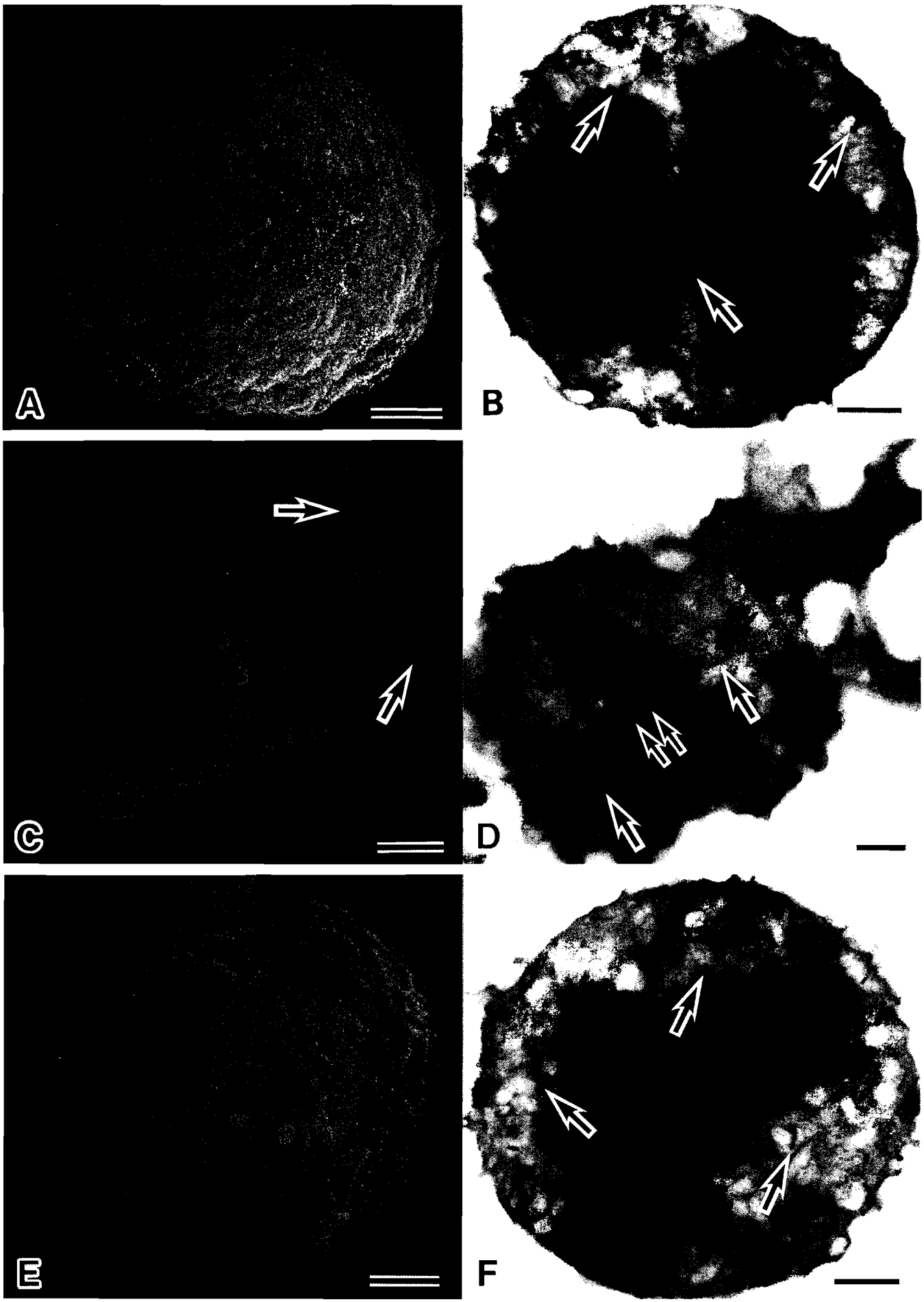
Results

Morphology of neutrophils and localization of ALPase activity

We examined the effect of rebamipide on morphology of human neutrophils and localization of ALPase activity, a marker of secretory granules. Using scanning and transmission electron microscopy, unstimulated human neutrophils showed a regular spherical outward appearance (Fig. 1A,B). Upon stimulation with FMLP, the cells revealed marked morphological alterations, such as elongated shape and presence of pseudopodia (Fig. 1C,D). No shape change was observed in cells loaded with rebamipide followed by stimulation with FMLP (Fig. 1E, F). In unstimulated neutrophils, ALPase activity was found in short rod-shaped intracellular granules distributed throughout the cytoplasm (Fig. 1B). After stimulation with FMLP, ALPase activity was seen in elongated tubular structures and vacuoles, indicating that secretory granules seen in unstimulated cells fuse to form larger structures upon stimulation (Fig. 1D). Following stimulation with FMLP, no reorganization of secretory granules was observed in cells exposed to rebamipide (Fig. 1F). Upon stimulation with PMA, neutrophils were relatively irregular in shape and revealed reaction product of ALPase activity in the same tubular structures and vacuoles as were observed in FMLP-stimulated cells. Morphological alterations were not detected in cells stimulated with PMA in the presence of rebamipide (data not shown).

Upregulation of ALPase activity and CD16

Association of secretory granules with the plasma membrane was studied in stimulated neutrophils by the measurement of ALPase activity and cell surface expression of CD16 (FcγRIII), another marker of secretory granules. In control, ALPase activity expressed to the cell surface was 2.7±0.8 nmol/30min/$1x10^6$ cells (mean±S.D.; n = 4) in unstimulated neutrophils. Rebamipide inhibited upregulation of ALPase activity in a dose-dependent manner (Fig. 2). In cells exposed to FMLP and to the mixture of rebamipide and FMLP, upregulation of ALPase activity was found to be 7.8±1.3 and 1.4±0.4 nmol/30min/$1x10^6$ cells (mean±S.D.; n = 4), respectively (Fig. 3), indicating that, upon stimulation with FMLP, 2 mM rebamipide inhibits 82.1% of upregulation of ALPase activity. In cells exposed to PMA and to the mixture of rebamipide and PMA, upregulated ALPase activity was found to be 7.5±0.4 and 7.7±1.0 nmol/30min/$1x10^6$ cells, respectively (Fig. 3), revealing that rebamipide does not inhibit upregulation of ALPase activity in cells stimulated with PMA. Then, we measured the cell


A
B
C
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F

Fig. 1. Scanning electron micrographs showing an outward appearance (A, C, E) of human neutrophils and transmission electron micrographs of thick (0.5 μm) sections of the cells demonstrating localization of the sites of ALPase activity (B, D, F). Neutrophils ($1x10^6$ cells/ml) were exposed to 2 mM rebamipide in PBS containing 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 mM glucose for 10 min at 37 °C followed by stimulation with 10^{-7} M FMLP for 5 min at 37 °C. **A, B.** Unstimulated cells are spherical and show ALPase reaction in short rod-shaped secretory granules (arrows). **C, D.** Cells stimulated with FMLP become elongated with pseudopodia (arrows in C) and contain ALPase reaction in tubular structures (double arrow) and vacuoles (arrows) formed by fusion of secretory granules. **E, F.** Cells exposed to the mixture of rebamipide and FMLP are spherical and ALPase reaction is seen in short rod-shaped secretory granules (arrows). Bars: 1 μm.

surface expression of CD16. The relative fluorescence intensity based on fluorescence intensity in cells exposed to FMLP (regarded as 100%) was 25.9±8.3, 91.0±12.0 and 88.5±16.8 (%±S.D.; n = 3) in cells exposed to the mixture of rebamipide and FMLP, to PMA, and to the mixture of rebamipide and PMA, respectively (Fig. 4). In control, the amount of cell surface expression of CD16 was 20.1±4.9 (%±S.D.; n = 3) in unstimulated cells. This result showed that rebamipide inhibited upregulation of CD16 (73.5% of inhibition) in cells exposed to FMLP, but not to PMA.

Chemotaxis

As mentioned above, rebamipide did not induce morphological changes of human neutrophils stimulated with FMLP. We attempted to elucidate whether it can influence the FMLP-induced migration of these cells. We employed a chemotaxis chamber partitioned by a micropored polycarbonate membrane and incubated cells for 10 min as described in Materials and Methods. The incubation time was critically important for counting the polycarbonate membrane-attached neutrophils migrating through micropores. Extended incubation (over 10 min) resulted in the detachment of cells from polycarbonate membrane and their migration into the lower chamber, thus decreasing the number of membrane-attached cells. Unstimulated cells showed no migration pattern under this incubation condition. Figure

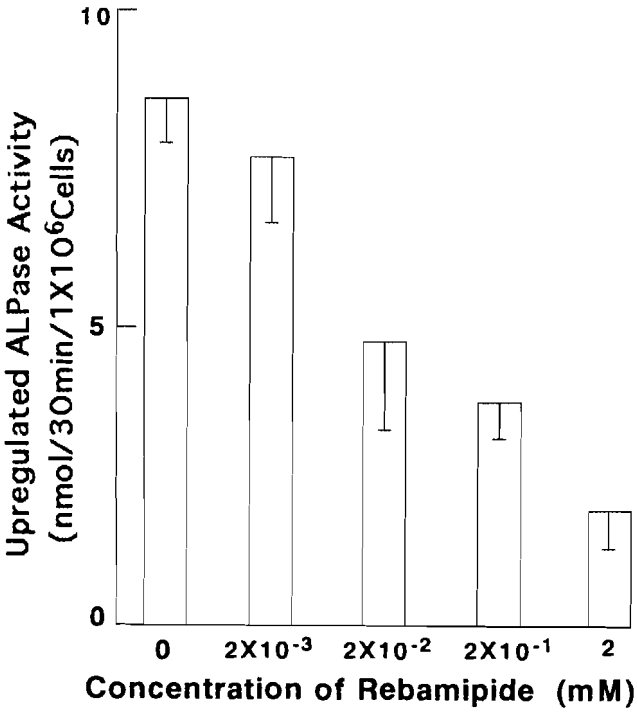

Fig. 2. Suppressive effect of rebamipide on upregulation of ALPase activity in human neutrophils stimulated with FMLP. Cells were exposed to various concentrations of rebamipide for 10 min at 37 °C followed by stimulation with 10^{-7} M FMLP for 5 min at 37 °C. Rebamipide inhibits upregulation of ALPase activity in a dose-dependent manner. The data represent the mean±S.D. from four separate experiments.

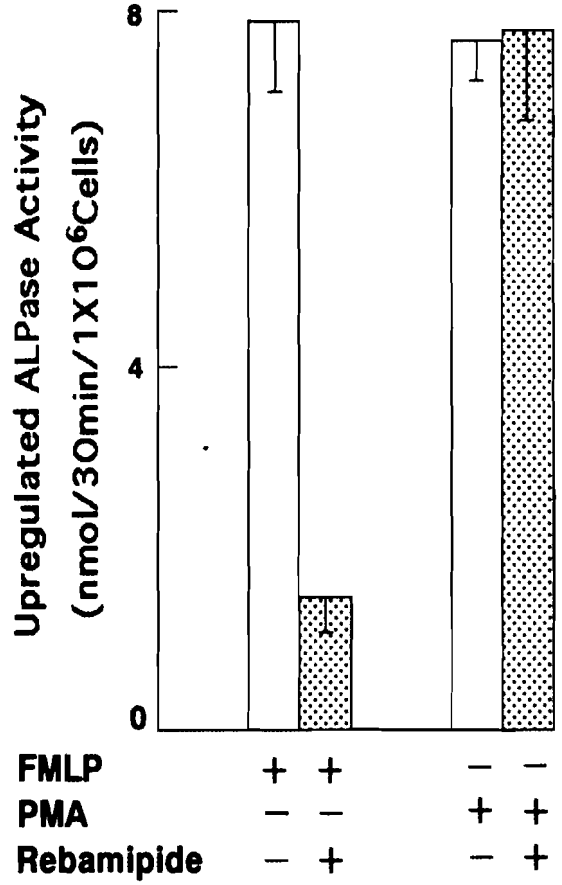

Fig. 3. Effect of rebamipide on upregulation of ALPase activity in human neutrophils stimulated with either FMLP or PMA. Cells were exposed to 2 mM rebamipide for 10 min at 37 °C followed by stimulation with 10^{-7} M FMLP or 50 ng/ml PMA for 5 min at 37 °C. Upon stimulation with FMLP, 2 mM rebamipide inhibits 82.1% of upregulation of ALPase activity. Following exposure to PMA and to the mixture of rebamipide and PMA, the upregulation of ALPase activity is not affected. The data represent the mean±S.D. from four separate experiments.

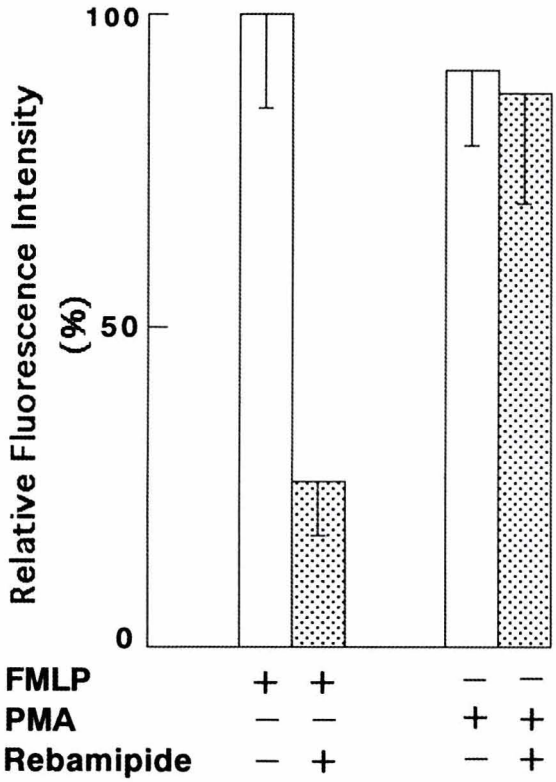

Fig. 4. Effect of rebamipide on upregulation of CD16 in human neutrophils stimulated with either FMLP or PMA. Cells were exposed to 2 mM rebamipide for 10 min at 37 °C followed by stimulation with 10^{-7} M FMLP or 50 ng/ml PMA for 5 min at 37 °C. The relative fluorescence intensity was calculated on the basis of fluorescence intensity in cells exposed to FMLP (regarded as 100%). Rebamipide inhibits 73.5% of CD16 upregulation in cells exposed to FMLP, but has little effect toward upregulation of CD16 in cells exposed to PMA. The data represent the mean±S.D. from three separate experiments.

5 is a micrograph of polycarbonate membrane-attached migrating cells taken by bringing the surface of the membrane facing the lower chamber in focus. Upon stimulation with FMLP, more neutrophils migrated to the membrane surface facing the lower chamber through the membrane pores (Fig. 5A) compared to the number of cells stimulated in the presence of rebamipide (Fig. 5B). In the latter case, we observed that migrating cells were unable to penetrate completely through micropores. Rebamipide inhibited FMLP-induced chemotaxis in a dose-dependent manner. The number of migrating cells counted in a random-sampling manner was determined as $1.41\pm0.35\times10^3$ and $0.06\pm0.06\times10^3$ cells/mm^2 (mean±S.D; n =4) in cells exposed to FMLP and to the mixture of rebamipide and FMLP, respectively (Fig. 6), meaning that inhibition rate of rebamipide on cell migration constituted 95.7%.

Intracellular calcium ion concentration ($[Ca^{2+}]_i$)

Changes in $[Ca^{2+}]_i$ concentration, which is a critical

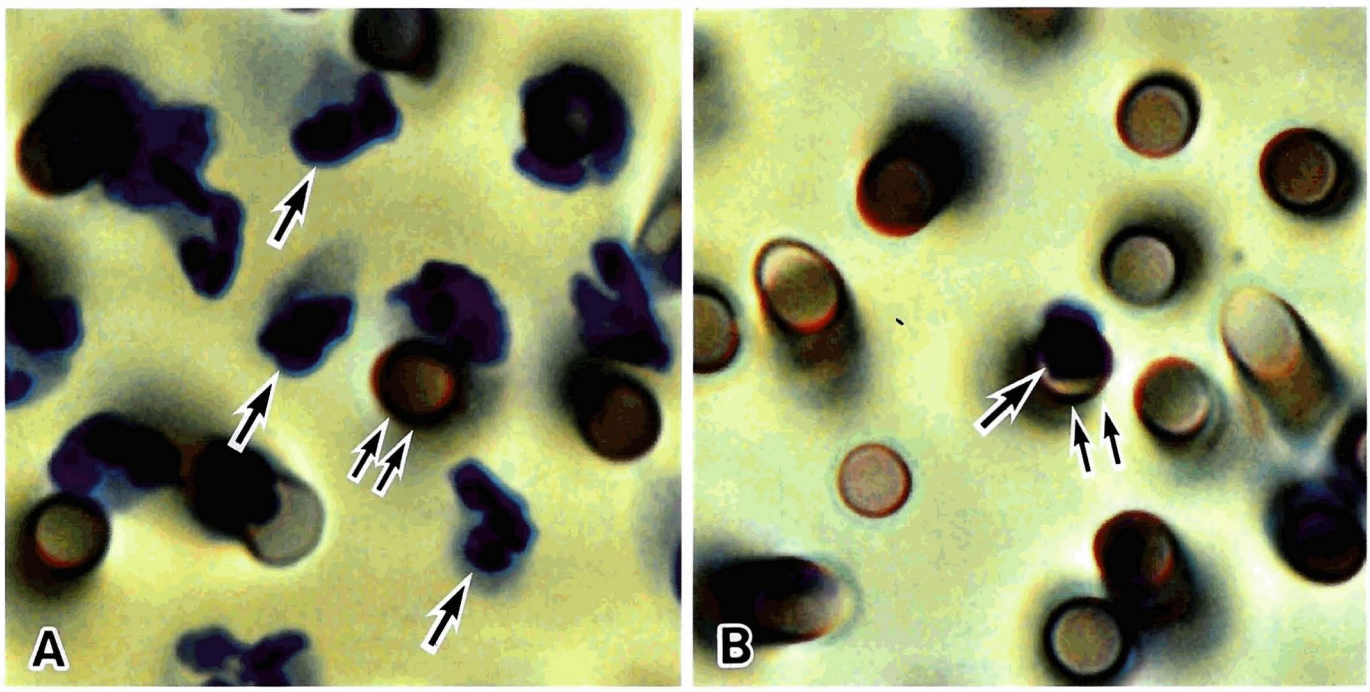

Fig. 5. Effect of rebamipide on chemotaxis of human neutrophils stimulated with FMLP. Chemotaxis was assayed using a chemotaxis chamber as described in Materials and Methods. **A.** In the absence of rebamipide, a large number of neutrophils (arrows) is seen migrating through micropores in membrane (double arrow). **B.** Rebamipide strongly inhibits the ability of neutrophils to migrate. Arrow shows a neutrophil unable to penetrate completely through the micropore in the membrane (double arrow).

signal in the process of mediation between ligand recognition and end response, are extremely important for various kinds of functional processes in neutrophils (for recent review see Pettit et al., 1997). We examined the effect of rebamipide on alteration in $[Ca^{2+}]_i$ concentration in neutrophils stimulated with FMLP. Stimulation of cells with FMLP caused a rapid increase in $[Ca2+]_i$ concentration reaching maximum at 794±120 nM (mean ±S.D; n = 3). After addition of FMLP, the concentration gradually decreased reaching 389±51 nM by 120 sec (cut off time). Exposure of cells to FMLP in the presence of rebamipide induced a slight increase in $[Ca^{2+}]_i$ concentration and a similarly gradual decrease in $[Ca^{2+}]_i$ concentration by 120 sec. The value of $[Ca^{2+}]_i$ concentration was 236±40 nM and 91±17 nM in the former and the latter case, respectively. The inhibition rate of rebamipide on the increase in $[Ca^{2+}]_i$ concentration was determined as 77.1% (Fig. 7).

Table 1. Effect of rebamipide on NADPH oxidase activation in human neutrophils stimulated with either FMLP or PMA.

TREATMENT*	ACTIVITY** (nmol/min/1x10^{6}cells)	TOTAL ACTIVITY (%)
FMLP	0.21±0.05	100
Rebamipide + FMLP	0.06±0.05	29
PMA	3.45±0.21	100
Rebamipide + PMA	3.29±0.37	95

*: cells were incubated in PBS containing Mg^{2+}, Ca^{2+} and glucose with or without 2 mM Rebamipide for 10 min at 37 °C followed by stimulation with either 50 ng/ml PMA or 10^{-7} M FMLP at 37 °C. **: activity was determined spectrophotometrically by superoxide dismutase-inhibitable reduction of ferricytochrome c at 550 nm. Data shown are the mean activity±S.D. from four separate experiments.

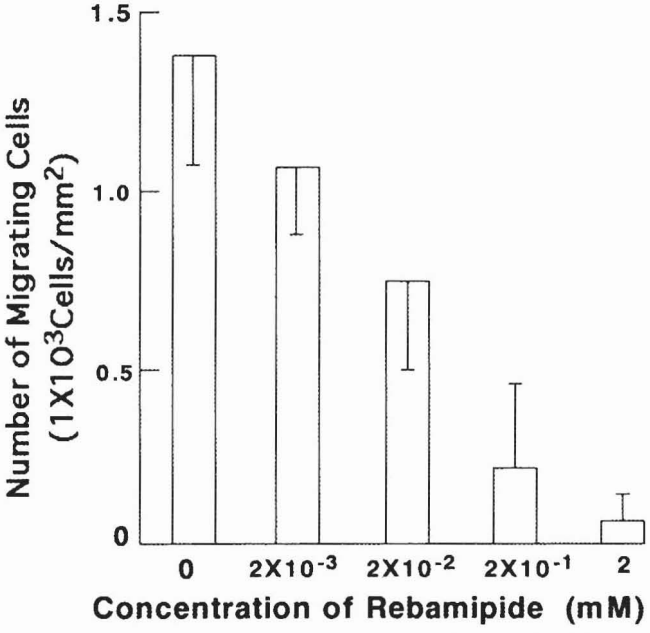

Fig. 6. Suppressive effect of rebamipide on chemotaxis of human neutrophils stimulated with FMLP. Chemotaxis was assayed using a chemotaxis chamber. Upper and lower chambers were filled with PBS, containing Mg^{2+}, Ca^{2+}, glucose and varying concentrations of rebamipide, as described in Materials and Methods. Inhibition rate of rebamipide on cell migration constitutes 95.7%. The data represent the mean±S.D. from four separate experiments.

Activation of NADPH oxidase

The effect of rebamipide on superoxide release in cells stimulated with either FMLP or PMA was studied by measuring the superoxide dismutase-inhibitable reduction of ferricytochrome c. NADPH oxidase activity was found to be 0.21±0.05 and 0.06±0.05 nmol/min/1±10^6 cells (mean±S.D.; n = 4) in neutrophils exposed to FMLP and to the mixture of rebamipide and FMLP, respectively (Table 1). Following exposure to PMA and to the mixture of rebampide and PMA, activity

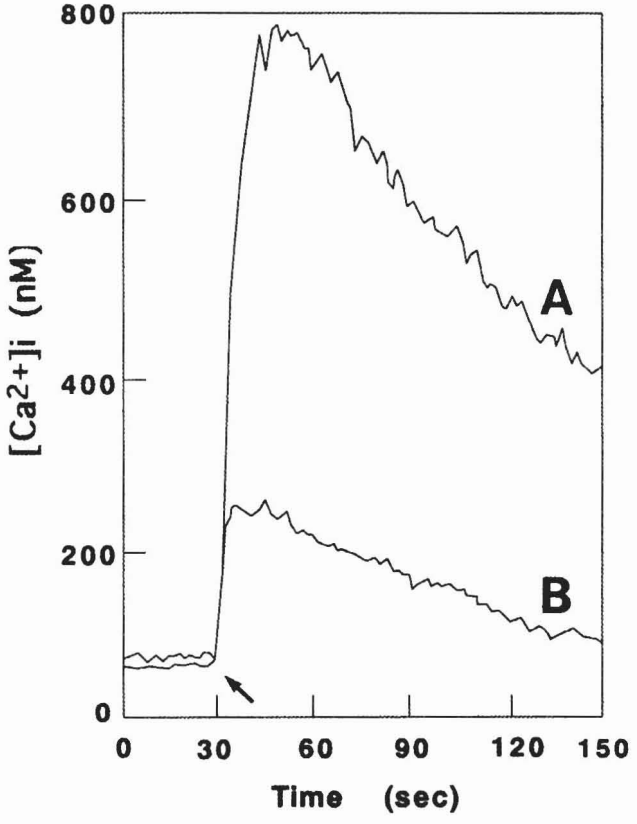

Fig. 7. Effect of rebamipide on FMLP-induced intracellular calcium ion ($[Ca^{2+}]_i$) concentration in Fura-2-loaded human neutrophils. Cells were incubated for 15 min at 37 °C in PBS containing Mg^{2+}, Ca^{2+}, glucose and 5 μM Fura 2-AM followed by exposure to 2 mM rebamipide. **A.** Stimulation with FMLP causes a rapid increase in $[Ca^{2+}]_i$ concentration, reaching maximum at 794±120 nM (mean ±S.D), and a slight decrease in $[Ca^{2+}]_i$ concentration reaching 389±51 nM by 120 sec (cut off time). **B.** Exposure of cells to FMLP in the presence of rebamipide induces a slight increase in $[Ca^{2+}]_i$ level and a similarly gradual decrease in $[Ca^{2+}]_i$ level by 120 sec. An arrow indicates addition of 10^{-7} M FMLP. Each graph is a representative of three separate experiments.

of the enzyme was detected as 3.45±0.21 and 3.29±0.37 nmol/min/1x10^6 cells, respectively (Table 1). These results demonstrate that rebamipide inhibited 71% of superoxide release in cells stimulated with FMLP. On the contrary, rebamipide was unable to affect the superoxide production in cells stimulated with PMA.

Discussion

The present study demonstrates that rebamipide, an antiulcer agent, influences several important biological functions of human neutrophils stimulated with FMLP, but not with PMA, and suggests that the upstream point of protein kinase C is the signal transduction pathway involved in its regulation.

To determine the effect of rebamipide on biology of human neutrophils, we evaluated the dynamics of ALPase-containing intracellular compartments. This type of granules contains several membrane proteins, namely membrane factor cytochrome b_{558} of NADPH oxidase, adhesion molecule CD11b, Fc-receptor CD16, and FMLP-receptor involved in inflammatory reaction (Borregaard et al., 1993; Borregaard and Cowland, 1997). In early phase of neutrophil activation, ALPase-containing granules are mobilized to cell surface during stimulation by mediators of inflammation (Borregaard et al., 1990; Sengeløv et al., 1993). This results in an immediate surface expression of cytochrome b_{558} (Calafat et al., 1993). Our data confirmed that rebamipide inhibits an intracellular reorganization of secretory granules, which fuse to form elongated tubular structures and vacuoles, concomitantly with the surface expression of markers of secretory granules, ALPase activity and CD16. This indicates that rebamipide acts to immobilize essential inflammatory membrane proteins and to prevent them from moving toward the cell surface. We found no morphological changes in cells exposed to the mixture of rebamipide and FMLP, although in the absence of rebamipide FMLP-stimulated cells became elongated in shape and showed pseudopodia.

We tested whether rebamipide is able to affect the chemotaxis in human neutrophils, since rearrangement of actin filaments is known to induce chemotaxis along with formation of pseudopodia upon cell stimulation (Moffat et al., 1996; Luna et al., 1997; Bernard et al., 1999; Middelhoven et al., 1999). We found that rebamipide inhibits FMLP-induced neutrophil migration, therefore suggesting that the drug influences rearrangement of cytoskeleton. Experiments are presently under way to elucidate whether rebamipide affects the rearrangement of actin filaments in human neutrophils stimulated with FMLP.

Since activation of N-formyl peptide receptor in human neutrophils by ligands, such as FMLP, induces an increase in $[Ca^{2+}]_i$ concentration together with chemotaxis (Miettinen et al., 1998), we examined the effect of rebamipide on an increase in $[Ca^{2+}]_i$ concentration. It was found that rebamipide inhibits an increase in $[Ca^{2+}]_i$ concentration by 77.1%, and that $[Ca^{2+}]_i$ concentration is gradually reduced to levels detected in unstimulated cells by 120 sec after stimulation with FMLP. These observations are in agreement with data obtained in experiments by Murakami et al. (1998).

Previously, we postulated that cytosolic factors of NADPH oxidase are associated with membrane factors localized in secretory granules and demonstrated that superoxide is released extracellularly by fusion of the granules to the plasma membrane (Kobayashi and Seguchi, 1999). These results are supported by the recent report of Telek et al. (1999). The authors used confocal laser scanning microscopy to elegantly demonstrate that the reactive oxygen species originate from intracellular compartments to be released extracellularly upon stimulation. To further confirm the previous observations, we show herein the suppressive effect of rebamipide on extracellular release of superoxide in cells stimulated with FMLP by measuring the reduction of cytochrome c (McCord and Fridovich, 1969). Employing methods based on different principles of detection, rebamipide was shown as an inhibitor of superoxide production in FMLP-stimulated cells in studies utilizing such assays like luminol-enhanced chemiluminescence and flow cytometry (Danielssonn and Jurstrand, 1998). The former measures amount of reactive oxygen species inside and released outside the cells (Fredlund et al., 1988), while the latter detects the amount of reactive oxygen species solely inside the cells (Perticarari et al., 1994). Findings of the present study, in agreement with the report mentioned above (Danielssonn and Jurstrand, 1998), demonstrate that rebamipide inhibits activation of NADPH oxidase in secretory granules and that superoxide is laborious to be released extracellularly even when the enzyme is partially activated in cells stimulated with FMLP in the presence of rebamipide, since rebamipide suppresses association of secretory granules with the plasma membrane.

To clarify the mechanism of rebamipide action, we examined the effect of the drug on neutrophil functions upon stimulation with PMA as compared to FMLP. We found that alterations in neutrophils morphology, mobilization of ALPase-containing secretory granules, upregulation of ALPase activity and CD16, and activation of NADPH oxidase are all suppressed by rebamipide in cells exposed to FMLP, but not to PMA. The latter is known to activate protein kinase C (Nishizuka, 1986) which is physiologically stimulated by diacylglycerol. The cell surface receptors including FMLP receptor are linked to heterotrimeric guanosine 5'-triphosphate-binding proteins which participate in the activation of phosphoinositide-specific phospholipase C. This phospholipase, in turn, generates diacylglycerol and inositol 1,4,5-triphosphate as second messengers of signal transduction (Boulay et al., 1990; Bokoch, 1995). Binding of inositol 1,4,5-triphosphate to Ca^{2+} stores induces the release of Ca^{2+} to the cytosol (Clapham,

1995). It is likely, therefore, that the upstream point of protein kinase C is involved in the suppressive action of rebamipide shown in this study. It was reported that rebamipide inhibits production of reactive oxygen species through the blocking of FMLP-related receptors (Danielsson and Jurstrand, 1998) and that it shows decrease in binding of FMLP to neutrophils (Kim and Hong, 1997). At least two mechanisms may therefore be considered with respect to the suppressive effect of rebamipide on neutrophil function: (1) the drug affects neutrophils through direct binding to FMLP receptor, and (2) it acts through inhibition of exocytosis of secretory granules by suppressive action to the point other than FMLP receptor, because secretory granules are the storage of FMLP receptors and deliver them to the cell surface upon stimulation.

In conclusion, results of the present study demonstrate that rebamipide inhibits exocytosis of secretory granules, chemotaxis, an increase in $[Ca^{2+}]_i$ concentration and release of superoxide in human neutrophils stimulated with FMLP, but not with PMA. It is suggested that the upstream point of protein kinase C is the signal transduction pathway involved in its regulation.

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